# Tear Copper and its Association with Liver Copper Concentrations in Six Adult Ewes

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## ABSTRACT

**Tear and liver copper concentrations from 6 clinically healthy adult mixed-breed ewes were measured by Atomic Absorption Electrothermal Atomization (graphite furnace) Spectrometry and Flame Absorption Spectrometry, respectively, 7 times over 227 d to determine if their tears contained copper and if so, whether tear copper concentrations could reliably predict liver copper concentrations. To produce changes in liver copper concentration, the diet was supplemented with copper at concentrations that increased from 23 mg to 45 mg Cu/kg feed/day/sheep during the study. This regimen raised liver copper for all sheep to potentially toxic hepatic tissue concentration of greater than 500 mg/kg dry (DM) matter (tissue).**

**The results of the study showed that copper was present in the tears of all sheep. The mean tear copper concentration showed a positive correlation with liver copper concentration ($P$ = 0.003), increasing from 0.07 mg/kg DM at the start to 0.44 mg/kg DM at the end of the study, but could not reliably predict liver copper concentration ($R^2$ = 0.222).**

## RÉSUMÉ

**Les concentrations lacrymales et hépatiques de cuivre ont été déterminées chez 6 brebis adultes cliniquement saines. Des techniques de spectrométrie par absorption atomique et par absorption de flamme ont été réalisées 7 fois sur une période de 227 jours dans le but de déterminer si les larmes contiennent du cuivre et si les concentrations lacrymales peuvent permettre de prédire adéquatement les concentrations hépatiques. Des niveaux croissants de cuivre ont été ajouté à l'alimentation des animaux afin d'induire un changement dans la concentration hépatique. Ce régime entraîna une augmentation des concentrations hépatiques de cuivre à des niveaux pouvant être potentiellement toxique pour le tissu hépatique.**

**L'étude à démontré que du cuivre pouvait être retrouvé dans les larmes. La concentration lacrymale moyenne en cuivre était corrélée positivement avec celle du foie ($P$ = 0.003), mais ne pouvait être utilisée comme moyen quantitatif de prédiction des concentrations hépatiques ($R^2$ = 0.222). (*Traduit par Dr Serge Messier*)**


## INTRODUCTION

The whole-body copper content of most healthy animals is approximately 2 mg/kg dry matter (DM) in the fat-free tissue (1). The concentration of copper in various body organs depends on the species, breed, diet, and the age of the animal. Ruminants have a high capacity for hepatic copper storage and sheep are reported to store from 72% to 79% of their total body copper in the liver (1). Chronically increased liver copper concentrations in sheep result in very few clinical signs (2). It is not until a copper induced hemolytic crisis occurs, often secondary to stress and/or compromised liver function, that the serum or plasma copper concentration significantly rises and the classical clinical signs of copper toxicity appear. Since serum or plasma copper concentration remains within the normal range (0.75–2.0 mg/L) during the period of hepatic accumulation of copper, prediction of a crisis during this period is not possible by serum or plasma analysis (3,4,5). Analysis of serum levels of liver enzymes are also not predictive tests. Liver copper concentration measured from a liver biopsy sample is the best sentinel, but not practical for routine surveillance (3,4).

To date, nonliver sentinels for impending hemolytic crisis are not available. Woolliams *et al* concluded that wool did not reflect increases in liver copper concentrations (6,7).

Our consideration of the possibility that sheep tears may contain copper and perhaps reflect liver copper status was based on two facts. Copper visibly accumulates in Descemet's membrane in humans whose liver copper concentrations are elevated and that the ocular tear film of people contains potentially copper binding proteins (8,9,10,11,12).

To test the hypothesis that tears contain copper and reflect liver copper status, paired tear and liver copper concentrations were determined serially over approximately an 8 mo period, from 6 sheep, whose diets were supplemented with copper. Copper supplementation was intended to increase liver copper concentrations over time; so if a relationship between liver and tear copper did exist, the correlation might be more apparent.

The purpose of this study was to determine if copper was present in the tears of these sheep and, if so, was there

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a predictable relationship between tear and liver copper concentrations.

## MATERIALS AND METHODS

### ANIMALS AND HOUSING

Six clinically healthy, 2–4 y-old, Suffolk-Targee, nongestating ewes, ranging in body weight from 60–109 kg, were housed together outdoors, with access to a wood-framed shelter. The experimental protocol was approved by the University of Wisconsin School of Veterinary Medicine Animal Care and Use Committee.

*Ration* — Each animal was fed approximately 2 kg/d of hay. The lot of hay was analyzed (13) (Soil and Plant Analysis Laboratory, Madison, Wisconsin) for copper, molybdenum and sulfur by Inductively-Coupled Plasma Emission (ICP) spectrophotometry per the Wisconsin Procedures for Soil Testing (Plant Analysis and Feed & Forage Analysis: No. 6 Soil Fertility Series 1970 (revised 1980, 1987), Department of Soil Science, College of Agricultural and Life Sciences, University of Wisconsin-Extension-Madison). Water was available free choice and was not tested for copper.

*Copper supplement* (4) — An aqueous solution of cupric sulfate (cupric sulfate anhydrous powder, Mallinckrodt # 4848, Lot 4848 KCEA, Mallinckrodt, Inc., Paris, Kentucky) was prepared to deliver 16.5 mg/L of available (copper formula weight 63.54 ÷ cupric sulfate [$CuSO_4$] formula weight 159.60 × 100 = 39.81% available copper) copper via daily drenching. The hay ration contained 6.62 mg/kg copper DM, which when combined with the supplement, resulted in each animal being administered 23.14 mg/L of copper orally each day at the start of the study. After 28 d the copper was increased to 28.65 mg/kg feed/d for 4 d, then 34.2 mg/kg feed/d for 91 d and finally 45.12 mg/kg feed/d for the remaining 104 d of the study (3,14).

### OBSERVATIONS

All animals were observed daily for clinical signs of acute hemolysis, inappetence, increased thirst, weakness, trembling, pallor, hematuria, icterus, and accelerated breathing.

### SAMPLE COLLECTION

Samples of tears, serum, and liver for copper concentrations, were taken before copper dosing (day 0) together with complete blood counts, serum chemistry profiles, and fecal flotations. On days 21, 42, 63, 107, 160, and 227 samples of tears, serum, and liver for copper concentrations, were again collected; and, every 3 wk, serum aspartate aminotransferase (AST) analyses were done to monitor for liver damage.

*Tears* — Tears were collected from both eyes of all animals 7 times during the 227 d study. Plain 47 µL capillary tubes (Chase Instruments Corp. Cat. No. 2502, Glens Falls, New York) were used that had been pretreated to remove any copper residue (15). The capillary tubes were stored individually in the treated test tubes, which were covered with Parafilm (American Can Co. Dixie/Marathon, Greenwich, Connecticut), and stored in an acid treated and air dried sealed plastic container.

Tears were collected in a capillary tube held between the thumb and index finger. These fingers were covered with new latex finger cots to reduce the chance of copper contamination. Animals were restrained with a halter, and the left eye was collected first. The lower eyelid was gently rolled out with thumb pressure. The tip of the capillary tube was placed close to the culde-sac near the medial canthus and held in a semihorizontal position and slowly advanced toward a pool of tears. Care was taken to avoid touching the eyelid margins, cilia, or facial hairs. Capillary action drew the tears into the tube. If the tear flow up the tube stopped, the tube was withdrawn and repositioned. Occasionally the tip would engage the conjunctival tissue and flow would stop, mucus would plug the tube, or the pool of tears would deplete. A new tear pool would usually form rapidly. At least one-half the volume of the capillary tube, approximately 20 µL, was required to obtain an adequate sample. After collection the tube was returned to its test tube, covered with Parafilm, and placed in the storage container.

*Blood* — Jugular venipuncture was used to collect whole blood for the previously described analyses. Blood for serum copper was drawn in commercially available evacuated serum vacutainer tubes (Vacutainer [no additive] Becton Dickinson Vacutainer Systems, Rutherford, New Jersey). The manufacturer stated that any copper residue present in the tubes would be far below normal serum copper concentration.

*Liver* — Liver samples were aseptically collected transcutaneously with a Tru-Cut (14 gauge × 6″, Travenol Lab., Inc. Deerfield, Illinois) Biopsy Needle after each animal received intramuscular xylazine (Rompun, 20 mg/cc, Mobay Corporation, Animal Health Division, Shawnee, Kansas), procaine penicillin G (Crysticillin, 300 A.S., Solvay, Mendota Heights, Minnesota) and local infiltration of 2% lidocaine (Elkins-Sinn, Inc., Cherry Hill, New Jersey) (16). Ultrasound was used to assist in locating the liver for sample collection in all animals where no sample was obtained on the first biopsy attempt. Liver was identified in the Tru-Cut needle by inspection. No attempt was made to remove any blood from the biopsy specimen because the level of copper in the liver was expected to far exceed the serum or plasma copper levels and therefore would not effect the results.

Test tubes that had been pretreated, etched with an identification number, and then carefully weighed (tare), were used as a receptacle for each liver sample.

### SAMPLE ANALYSIS

*Tears* — Copper determinations for tears were done by Atomic Absorption Electrothermal Atomization (graphite furnace) Spectrometry (EA) (Central Animal Health Diagnostic Laboratory, Madison, Wisconsin). The more sensitive graphite furnace method was chosen for tear analysis because of the small sample size and anticipated low concentration of tear copper.

Ten microliters of tears were diluted with 90 µL of deionized water (DI) in a fresh pretreated tube using an automatic pipette. A blank (DI) and standard (0.05 µg/mL copper) were run in pretreated tubes. The

machine was auto-zeroed after the blank was run to subtract any residual copper contamination. Each tear sample was run 4 times and the absorbance values obtained were averaged and used to calculate copper concentration using a ratio ([0.05($mean_T$ × 10)]/$mean_s$ = μg/mL Tear Copper: where T = EA absorbance units for tears and S = EA absorbance units of the Standard). The mean of the tear copper concentrations in μg/mL are reported as mean tear copper (MTC).

*Blood* — Serum copper determination was done (within 24–48 h) by means of Flame Atomic Absorption Spectrometry (FAS). At that time serum was diluted 1:5 with DI, and a blank (DI) and a standard (1.0 μg/mL copper) were also run with each batch. Serum copper (SC) was reported in mg/L.

*Liver* — After wet weight was determined the liver samples were air dried overnight at 105°C, placed in a desiccator for 30 min, and then reweighed to obtain the dry weight. After the sample was ashed (Muffle Furnace @220°C for 1 h followed by 1°/min ramp to 550°C for 6 h), the copper in the sample was brought into solution by means of hydrochloric and nitric acid and each sample was reconstituted to a volume of 10 mL. The samples were analyzed for copper by FAS. The FAS was fitted with a hollow cathode lamp (flame wave length = 324.7 nm and 0.7 nm spectral band width) and a laboratory copper standard of 5.0 μg/mL was used to standardize the spectrometer. The samples were aspirated into the FAS which automatically generated the mean of 3 absorbance readings in μg/mL units. These mean values were used to calculate (FAS mean value μg/mL × 10 mL)/net dry weight of sample in grams = mg/kg DM liver copper) liver copper concentrations in mg/kg DM of the liver biopsy samples (liver copper concentration dry matter (tissue) = LCDM) (16).

*Data analysis* — The Multivariate General Linear Models Procedure of Systat (Wilkinson, Leland. SysStat: The System for Statistics. Evanston, Illinois; SysStat, Inc., 1990) was used to test for linear relationships between tear copper and liver copper and for differences in slopes and/or intercepts among the study sheep. Additionally, we plotted the residuals of the LCDM vs MTC data against estimated values. Rankit plots were used to assess the fit of the model, homoscedasticity, and normality of the data.

To assess the validity of the mean of the tear copper value from the right and left eye, a paired two tailed *t*-test was performed on the left (TCOS) and right (TCOD) eye tear copper data. All graphs and the regression in Fig. 3 were done using SigmaPlot's (Jandel Scientific, San Rafael, California) graphical and statistical options.

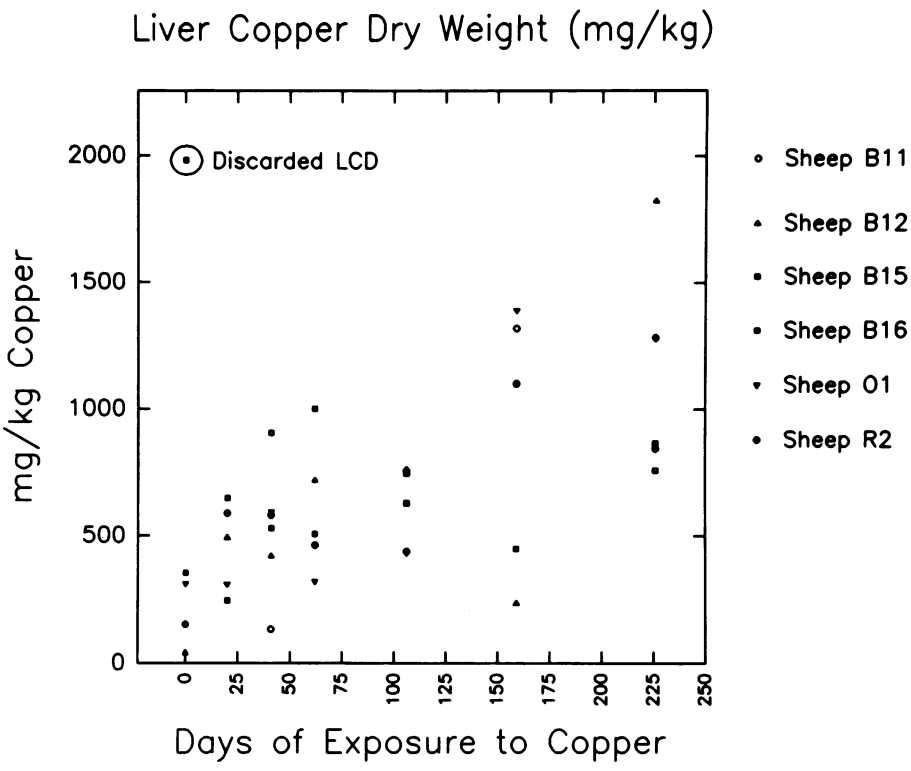


**Fig. 1. Range of liver copper concentrations in mg/kg dry weight for each study sheep at each collection interval over the 227 d study period. The liver copper dry (matter) LCDM erroneous value for sheep B16 that was discarded is circled.**

## RESULTS

The study animals remained clinically healthy. Copper, molybdenum and sulfur in the hay ration (DM) were 6.62 mg/kg CU, 0.57 mg/kg MO and 0.25% S. Prestudy CBC, chemistry panel, and fecal analyses were normal. The serum AST remained within normal limits. Insufficient liver biopsy samples were obtained from R2 on study days 0, 21, and 63. Animal B16 had elevated LCDM concentration of 1981 mg/kg on day 0 and its serum copper was 1.46 mg/L. This measurement was assessed as erroneous and not included in the final data analysis. The right eye tear sample from animal O1 on day 107 was lost.

The range of liver biopsy sample dry weight was between 0.0015 gm – 0.0782 gm with a mean weight of 0.0165 gm and SD of 0.0125 gm. Baseline liver biopsy copper concentrations for all 6 sheep ranged from 40.34 mg/kg – 353.2 mg/kg DM (mean 213.75 mg/kg DM; median 230.74 mg/kg DM; standard deviation (SD) 144.34 mg/kg DM); MTC concentrations ranged from 0.05 μg/mL – 0.11 μg/mL (mean 0.07 μg/mL; median 0.07 μg/mL; SD 0.02 μg/mL); and serum copper concentration ranged from 0.87 mg/L – 1.46 mg/L (mean 1.26 mg/L; median 1.35 mg/L; SD 0.23 mg/L).

Changes in liver and tear copper concentrations from all animals over the course of the study are summarized in Figs. 1 and 2. The LCDM ranged from 40.34 mg/kg – 1823.5 mg/kg DM, the MTC ranged from 0.04 μg/mL – 0.93 μg/mL, and the serum copper concentration ranged from 0.65 mg/L – 1.93 mg/L. The LCDM range extended

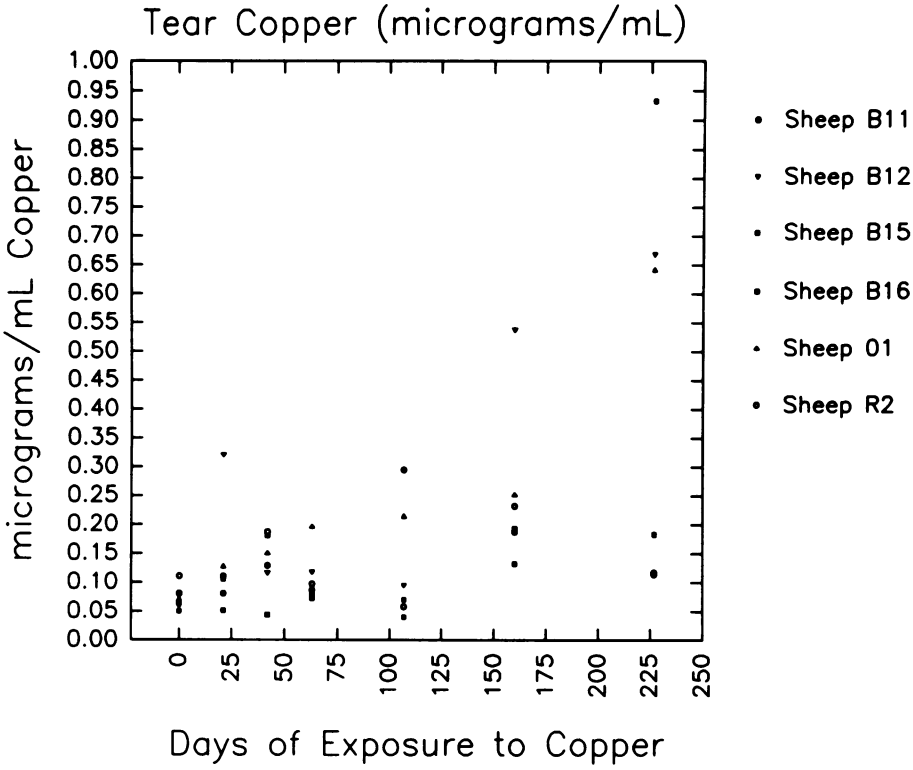


**Fig. 2. Range of tear copper concentrations (mean value for both eyes) in μg/mL for each study sheep at each collection interval over the 227 d study period.**

from low normal to concentrations published for advanced stages of chronic copper poisoning, and the serum copper in general stayed within the normal published range for sheep.

There was a significant increase in LCDM with time among the study animals ($P < 0.0001$), Fig. 1. The mean LCDM increased from 213.75 mg/kg DM at the baseline to a mean of 1142.01 mg/kg DM (median 1072.3 mg/kg; SD 403.87 mg/kg) at the end of the study. The MTC concentration increased from a baseline value of 0.07 μg/mL to 0.44 μg/mL (median 0.41 μg/mL; SD 0.35 μg/mL) by the end of the study, and there was a correlation between MTC concentration and LCDM ($P = 0.003$, $\beta =$ 983.5 (mg/kg) LCDM/(μg/mL DM) MTC, $Y$ intercept = 483.523 mg/kg DM), Fig. 3, but not to a reliably predictable degree ($R^2 = 0.222$). Overall there was no significant difference in slopes or intercepts among sheep ($P >$ 0.05) with regard to the linear model relationship used in the analysis for LCDM vs MTC. These data were normally distributed and homoscedastic.

## DISCUSSION

Copper was detected in the tears of all sheep, but analysis of the data showed that the tear copper concentration could not reliably predict the concentration of liver copper. This is similar to the findings of others for other potential sentinels of elevated liver copper concentration such as hair and serum or plasma (3,6,7).

The serum copper in general stayed within the normal published range for sheep. The LCDM showed an increase in concentration over the study period that extended from low normal to concentrations published for advanced stages of chronic copper poisoning. These increases were interpreted as a response to the copper supplemented diet. There was some variation in LCDM and MTC concentrations, Figs. 1–3 amongst the study sheep over the 227 d study. Possible reasons for LCDM concentration variation could have been contamination with copper, laboratory error, variation in the distribution of copper throughout the liver, and small sample size. Contamination in any analytical procedure is possible; however, because glassware was chemically treated, any contamination would likely have come from other sources during collection or processing. Laboratory precision for the FAS is ±1 to 1.5% of the sample mean absorbance units (AU) for the laboratory where the analyses were done. With the small biopsy sample, weighing error could have accounted for inaccurate copper determination. In spite of the small liver biopsy sample sizes, we felt that the method was both precise and accurate, especially because an accurate electronic balance was used and because the liver samples were kept in the same decoppered tube through ashing. Diluting error could also account for variations, in spite of the use of volumetric flasks and automatic pipettes.

The literature is equivocal with regard to the distribution of copper in sheep liver. Hepatic copper concentration in sheep, particularly when the liver copper has reached dangerously high concentrations, varies considerably from one part of the liver to another (18). These authors recommended that, at necropsy, samples be taken from the caudate lobe, which was purported to have the highest copper concentration; however, they did not substantiate this claim in their report, nor did they reference their previous caudate lobe findings. The recommendation to use the caudate lobe for copper analysis is still in recent textbooks, all of which refer to the same paper (18). Clinically, the caudate lobe is not accessible by percutaneous biopsy. Other investigators have found uneven distribution of copper and iron in the pig liver both within and between lobes and have suggested that if variations were similar in other domestic animals, liver biopsy for mineral studies could give misleading results (19). A more recent study, on the other hand, has shown that the concentration of liver copper in lambs increases from the dorsal (right) lobe to the ventral (left) lobe, yet in adult sheep this trend was found to be less apparent or absent (20). Their conclusion was that the percutaneous biopsy technique provides a satisfactory sample, and this conclusion

served as the basis for our study protocol of one biopsy per sample interval and not the mean of multiple biopsies from different sites. If liver copper distribution is really highly variable, then the mean of samples from several sites would be appropriate, and could, in part, explain the one higher initial LCDM value and the variability of subsequent samples.

MTC does not reliably predict liver copper concentration yet all sheep had similar trends; ie: there was no significant difference in slopes or intercepts among sheep ($P > 0.05$). Sampling technique, contamination, small sample size and analysis error could account for some of the variation seen in the tear copper concentrations. The use of the MTC was appropriate, for when confounding effects of sheep-to-sheep and collection date-to-collection date variation were removed by using the sheep and collection date as a criteria for pairing, the results obtained for TCOS and TCOD were not consistently different. The distribution of differences between the 2 eyes were normally distributed about zero and therefore it also does not appear ($P = 0.636$; $T = 0.477$) that sampling 1 eye first affected the value obtained for the 2nd eye, because the order of sampling was always the same. Therefore MTC was used in the analysis.

The accuracy of the EA method was controlled by calibration of the EA instrument with a standard copper solution. The precision for the EA method in general for the laboratory that ran the analysis was about ±5% of the mean absorbance value, under perfect conditions. The sample coefficient of variation for the EA tear absorbance was 10%, which suggests less precision than the ±5% for the laboratory. The most common reason for EA sample error with small volume samples is pipetting error.

Any concern that tear copper was not being measured, was allayed, because the blanks, standards, and tears were all contained in decoppered tubes and the EA unit was autozeroed after each blank was run. Because EA for copper does not require any extraction or physical transformation of the sample matrix, and because copper is not volatile nor lost during the analysis

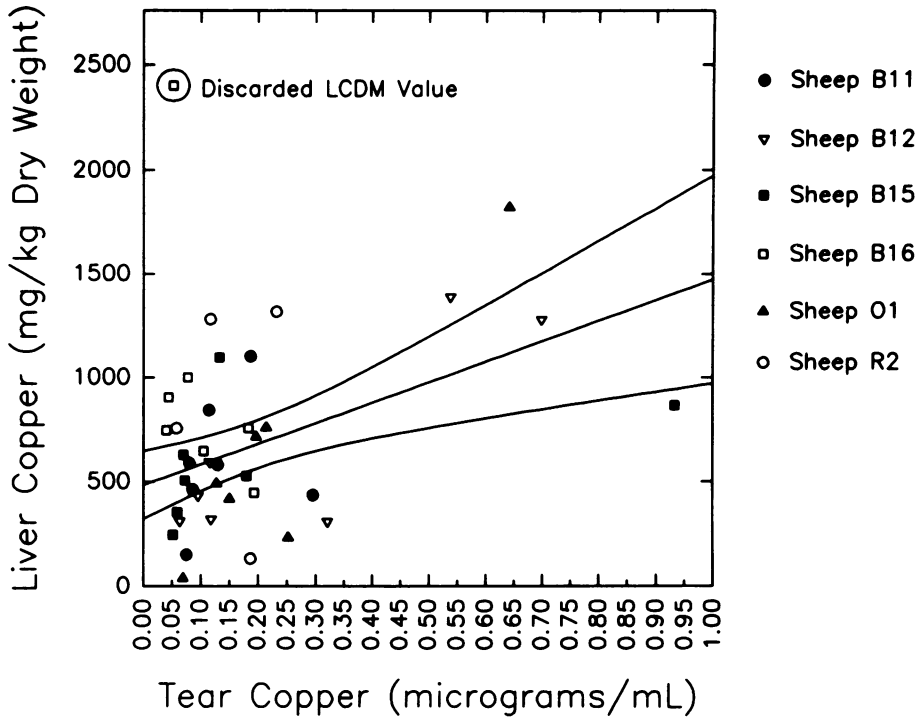


**Fig. 3. Relationship of liver copper concentrations in mg/kg dry weight vs corresponding tear copper concentration (mean value for both eyes) in μg/mL for each study sheep over the 227 d study period with a least-squares best fit analysis with 95% confidence intervals drawn to the axes. The discarded erroneously initial high liver copper dry (matter) LCDM value for study sheep B16 is shown to give a sense of its potential effect on the relationship had it been included in the analysis.**

even if bound to analyte matrix; loss of copper during the analysis was not likely. Tear copper concentrations were measured to a precision of 10%.

The strength of the statistically significant relationship between LCDM and MTC may be weaker, because the regression is influenced by 4 data points (Fig. 3). Two of these paired data points (Sheep B12 and B15; study day 227) had disproportionate respective amounts of tear copper in one eye (1.1 μg/mL and 1.69 μg/mL) as compared to the opposite eye (0.15 μg/mL and 0.18 μg/mL). The high MTC is influenced by the higher measurement. Confounding factors, previously discussed could be responsible for an erroneous high value. The lower values for the opposite eye were more reliable, because they were within the range of values obtained during the study. Since there is no information in the literature about tear copper and expected tear copper concentrations, no tear copper data were discarded. If the 2 high MTC concentrations are erroneous then the slope of the regression for LCDM vs MTC is strongly influenced by only 2 data points, which implies an improbable correlation between LCDM and MTC despite statistical correlation. One could argue that in total, there are relatively few data points, and perhaps if many more animals were measured, a clearer relationship between LCDM and MTC would appear. Our opinion is that even if a stronger statistical relationship were found, the weak predictive trait would still be present.

In conclusion, copper is present in the ocular tear film of sheep and appears to be affected by dietary copper intake; however, the amount of copper present in sheep tears does not reliably reflect liver copper status.

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